


# Best Available Copy

Serial No. 10/038,984  
Group No. 1635  
Confirmation No. 9705

③  **Europäisches Patentamt**  
**European Patent Office**  
**Office européen des brevets**

⑪ Publication number:

**0 286 224**  
**A2**

⑫ **EUROPEAN PATENT APPLICATION**

⑲ Application number: 88301824.4

⑳ Date of filing: 02.03.88

⑮ Int. Cl.<sup>4</sup>: **A61K 45/06 , A61K 31/70 ,**  
**A61K 45/02 , A61K 31/71 ,**  
**/(A61K45/06,31:70),**  
**(A61K45/02,31:70),(A61K31/71,**  
**31:70),(A61K31/70,31:66)**

⑳ Priority: 23.03.87 US 28823  
25.11.87 US 125097

㉑ Date of publication of application:  
12.10.88 Bulletin 88/41

㉒ Designated Contracting States:  
**AT BE CH DE ES FR GB GR IT LI LU NL SE**

㉓ Applicant: **HEM RESEARCH, INC.**  
12220 Wilkins Avenue  
Rockville Maryland 20852(US)

㉔ Inventor: **Carter, William A.**  
1 Jaine Lane  
Birchrunville Pennsylvania(US)

㉕ Representative: **Votier, Sidney David et al**  
**CARPMAELS & RANSFORD 43, Bloomsbury**  
**Square**  
**London WC1A 2RA(GB)**

㉖ **Treatment of human viral infection by dsRNA combined with viral inhibitors.**

㉗ Synergistic combinations of dsRNAs and anti-viral agents in the treatment of viral diseases indicates dsRNA, especially mismatched dsRNA, plays a powerful and versatile role as a core drug in combination therapy for human viral pathogens, notably for ARC and AIDS.

**EP 0 286 224 A2**

## TREATMENT OF HUMAN VIRAL INFECTIONS by dsRNA COMBINED WITH VIRAL INHIBITORS

### BACKGROUND OF THE INVENTION

This invention relates to the use of dsRNA in synergistic combination with other materials that inhibit viral activity or expression to control or in the treatment of viral diseases. Pharmaceutical compositions useful in such treatments are also disclosed.

Double-stranded RNAs (dsRNAs), such as poly I • poly C, can act as biological response modifiers eliciting antiviral, antineoplastic and immunomodulatory activities. Among the pleiotropic effects responsible for these biological responses are induction of interferon (IFN) and other cytokines as well as activation of certain INF-induced enzymes including 2,5-oligoadenylate synthetase and a ribosome-associated protein kinase. These properties make dsRNAs attractive candidates for the treatment of infection by human immunodeficiency virus (HIV), the retrovirus responsible for acquired immunodeficiency syndrome (AIDS). In fact, mismatched dsRNA of the form  $r(I)_n \bullet r(C:2 \cdot 4.U)_n$  or Ampligen® (a registered U.S. trademark of HEM Research, Inc., Rockville, Maryland USA) has a low toxicity profile in humans, is active against HIV infection both *in vitro* and *in vivo*, and is currently in large-scale, controlled clinical trials of AIDS-related complex (ARC).

The American Foundation for AIDS Research (AmFAR) currently lists over 60 drugs being tested for use in treating ARC and AIDS. This enormous potential of single-agent therapy is compounded by possible synergism in combination therapy. Alternatively, combined therapy has the potential for antagonism as demonstrated *in vitro* with azidothymidine (AZT) and ribavirin. For these reasons, I decided to characterize the full potential of mismatched dsRNA in the treatment of ARC and AIDS, HIV used as a prototypic viral pathogen. I accomplished this by performing *in vitro* multiple drug analyses using mismatched dsRNA as a core drug in combination with other agents that together encompassed at least five different modes of attack on this virus. These agents included rIFN-alpha A, rIFN-beta Ser 17 and rIFN- $\gamma$  as cytokines; azidothymidine and phosphonoformate (foscarnet) as inhibitors of reverse transcription; ribavirin as a putative disrupter of mechanisms governing proper mRNA capping; amphotericin B as a lipid-binding molecule with anti-HIV activity; and castanospermine as an inhibitor of glycoprotein processing (1). One of the drugs, azidothymidine, acted synergistically *in vitro* with mismatched dsRNA as described in more detail below. I have discovered that each of these drugs drug separately had dose-dependent anti-HIV activity which was synergistic with mismatched dsRNA at the most effective doses.

### DESCRIPTION OF THE INVENTION

This invention includes the use of synergistic combinations of dsRNAs, notably mismatched dsRNAs, together with a member of a wide range of antiviral compounds in the treatment of viral diseases. The combination is administered to a patient in an amount sufficient to inhibit viral activity, inhibit viral expression, or both. Pharmaceutical compositions containing a dsRNA and another antivirally-active compound are described and the results of this combination demonstrating synergism reported below.

Data are here presented verifying the role of dsRNA as a synergistic agent with various other modalities in control of viral expression in general and retroviruses in particular utilizing HIV (AIDS virus) as a prototypic human virus associated with chronic debilitating human disease. The invention includes therapies for susceptible viral infections other than HIV which have a common mechanism of viral multiplication/pathogenesis, in whole or in part, and accordingly are sensitive to the particular combination employed. Synergism in inhibiting viral activity/expression is unexpectedly seen with a broad range of disparate compounds including cytokines, reverse transcriptase inhibitors, lysophiles and glycoprotein processing inhibitors and the like antiviral compounds.

The effective treatment of AIDS has obviously become a growing concern among physicians in nearly all countries around the globe. Azidothymidine, the first drug approved in the United States for the treatment of ARC and AIDS, is extremely toxic. This *in vivo* toxicity is manifested by approximately 30% of patients receiving azidothymidine requiring blood transfusions. The results of my present experiments suggest that drugs like azidothymidine with high *in vivo* toxicity can be given at substantially lower, less toxic doses if combined with mismatched dsRNA. Combination therapy may not only reduce the effective dose of a drug required for antiviral activity, thereby reducing its toxicity, but may also improve the absolute antiviral effect as a result of attacking the virus through multiple mechanisms. The pleiotropic activities of mismatched

dsRNA together with the synergies reported here suggest that dsRNA in general, and mismatched dsRNA in particular, will be an effective core drug for combination therapy yielding the most effective and least toxic treatment for ARC and AIDS.

Studies with eight different antiviral drugs, representing five different general classes of antivirals, in combination with dsRNA, especially the mismatched dsRNAs as defined in more detail below, demonstrate that dsRNAs, notably mismatched dsRNAs, provide a synergistic complement to antiviral therapy in general, and in the treatment of HIV infections including AIDS-related complex (ARC) and AIDS itself specifically. These dsRNAs, when administered in combination with other antiviral drugs known to cause significant toxicity when administered alone and in quantities effective to address the viral condition, have the additional benefit of permitting the clinician to reduce the amount of the toxic member of the combination without adverse affect on the desired therapeutic results of the treatment.

To demonstrate the synergism of the combinations of this invention, multiple drug effect analyses with mismatched double-stranded RNA (Ampligen) as a core drug were performed to identify other agents and mechanisms through which mismatched dsRNA may potentiate effective therapeutic intervention in human immunodeficiency virus (HIV) infection. Antiviral activities were defined by a microtiter infection assay utilizing MT-2 cells as targets and HTLV-III<sub>B</sub> produced in H9 cells as a virus source. The scope of agents tested included rIFN- $\alpha$  A, rIFN- $\beta$  SER 17, and rIFN- $\gamma$  as cytokines; azidothymidine and phosphonoformate (Foscarnet) as inhibitors of reverse transcription; ribavirin as a putative inhibitor of proper HIV mRNA capping; amphotericin B as a lipophile; and castanospermine as a glycoprotein processing (glucosidase I) inhibitor. Separately, each drug demonstrated dose-dependent anti-HIV activity and, when used in combination with mismatched dsRNA, demonstrated synergism.

Although mismatched dsRNA was synergistic with all three IFNs for anti-HIV activity in microtiter infection assays, it did not potentiate IFN-induced inhibition of virus production in cultures of H9/HTLV-III<sub>B</sub> cells. The results of these studies suggest that the pleiotropic activities of dsRNAs differ from those of IFN and may provide synergism in combination therapy with a wide range of antiviral drugs for the treatment of AIDS.

The procedures and therapeutic compositions of this invention are intended to include the above-listed agents, as exemplary and illustrative of various classes there named. Various other agents yet to be discovered but synergistic when combination with dsRNAs are also included within the scope of this invention.

By "mismatched dsRNAs" are meant those in which hydrogen bonding (base stacking) between the counterpart strands is relatively intact, i.e., is interrupted on average less than one base pair in every 29 consecutive base residues. The term "mismatched dsRNA" should be understood accordingly.

The dsRNA may be a complex of polyinosinate and a polycytidylate containing a proportion of uracil bases or guanidine bases, e.g., from 1 in 5 to 1 in 30 such bases (poly I • poly (C<sub>4-29</sub> x > U or G). The dsRNA may be poly I • poly C,U in which the ratio of C to U is about 12-14 to 1, preferably 13:1, and the sedimentation coefficients of poly I and poly C,U are both less than 9 and within 2 units of each other; both are preferably about 6.5 to about 7.5.

The mismatched dsRNAs preferred for use in the present invention are based on copolynucleotides selected from poly (C<sub>n</sub>G) in which n is an integer having a value of from 4 to 29, and are mismatched analogs of complexes of polyriboinosinic and polyribocytidilic acids, formed by modifying rI<sub>n</sub>.rC<sub>n</sub> to incorporate unpaired bases (uracil or guanidine) along the polyribocytidylate (rC<sub>n</sub>) strand. Alternatively, the dsRNA may be derived from poly (I). poly (C) dsRNA by modifying the ribosyl backbone of polyriboinosinic acid (rI<sub>n</sub>) e.g., by including 2'-O-methyl ribosyl residues. These mismatched analogs of rI<sub>n</sub>.rC<sub>n</sub>, preferred ones of which are of the general formula rI<sub>n</sub>.r(C<sub>11-14</sub>U)<sub>n</sub> and rI<sub>n</sub>.r(C<sub>29</sub>G)<sub>n</sub>, are described by Carter and Ts'o in U.S. Patents 4,130,641 and 4,024,222. The dsRNAs described therein generally are suitable for use according to the present invention.

Specific examples of mismatched dsRNA for use in the invention include:-

- poly (I) • poly (C<sub>4</sub>U)
- poly (I) • poly (C<sub>7</sub>U)
- poly (I) • poly (C<sub>13</sub>U)
- poly (I) • poly (C<sub>22</sub>U)
- poly (I) • poly (C<sub>20</sub>G)
- poly (I) • poly (C<sub>29</sub>G) and
- poly (I) • poly (C<sub>p</sub> 23 G>p

The amount of mismatched dsRNA administered is preferably sufficient to achieve a peak blood

concentration of from 0.1 micrograms per milliliter of dsRNA up to 1000 micrograms per milliliter in the systemic blood circulation immediately following administration distal from the point of infusion. The dsRNA is administered parenterally (intravenously, intramuscularly, subcutaneously), intranasally, rectally or orally when suitably protected against the nucleases of the gastrointestinal tract.

5 When interferon (alpha) is used as the lymphokine, an amount of from 0.01 to 100,000 IRU per milliliter of the patient's body fluid is provided. When both agents (a dsRNA and another antiviral compound) are administered they may be administered as a mixture, administered separately but simultaneously, or sequentially. The complementary antiviral used with the dsRNA is administered in quantities consistent with the product labeling or other directions for use and often in somewhat smaller amounts, frequently  
10 substantially reduced, due to the concurrent use of the dsRNA and the synergistic result of the combination.

Administration of a dsRNA and another antiviral agent "in combination" includes presentations in which both agents are administered together as a therapeutic mixture, and also procedures in which the two agents are administered separately but simultaneously, e.g., as through separate intravenous lines into the same individual. Administration "in combination" further includes the separate administration of one of the  
15 drugs in which one of the drugs is given first followed shortly by the second.

A group of *in vitro* studies were undertaken to evaluate Ampligen, a mismatched dsRNA, for combination therapy in the treatment of viral diseases utilizing HIV as a prototypic chronic-subacute human viral pathogen. The materials and methods used are described below.

**Cells and Virus** -A clone of the HTLV-I-transformed T-cell line MT-2, which exhibits complete cytolysis  
20 upon infection with HIV, was used as the target for infections in microtiter assays. Virus was prepared from H9:HTLV-III<sub>a</sub> culture fluids by low speed centrifugation and 0.45  $\mu$ M filtration to remove all cells. Viral titers were determined from 50% tissue culture infectious dose (TCID<sub>50</sub>) values obtained by endpoint microtitration on MT-2 cells. All cultures were grown and maintained in RPMI-1640 containing 16% heat-inactivated fetal calf serum and 50  $\mu$ g gentamicin (Sigma)/ml.

25 **Antivirals** -Human rIFN- $\alpha$ A ( $>10^8$  IU/mg), rIFN- $\gamma$  ( $1.4 \times 10^8$  IU/mg) and azidothymidine were obtained from Hoffman-La-Roche. Human rIFN- $\beta$  Ser 17 ( $1.0 \times 10^8$  IU/mg) was obtained from Triton Biosciences. IFNs were calibrated in WISH cells challenged with vesicular stomatitis virus and assayed for cytopathic effect a previously described (2). Reference standards were obtained from the World Health Organization (human IFN- $\alpha$ , WHO standard B,69/19 and human IFN- $\beta$ , WHO no. G-023-902-527) or the National Institute  
30 of Allergy and Infectious Disease (human-IFN- $\gamma$ , National Institutes of Health no. Gg23-901-530). Amphotericin B (Fungizone) was obtained from GIBCO, castanospermine from Boehringer Mannheim, ribavirin (Virazole) from Viratel, Inc., 3'-azido-3' deoxythymidine (AZT, Retrovir), research grade, from Roche Laboratories, and phosphonoformate (Foscarnet) from Astra Alab AB. Mismatched dsRNA (Ampligen) was provided as a lyophilized powder in a salt buffer by HEM Research, Inc., Rockville, Maryland.

35 **Microtiter Infection Assay** -Anti-HIV activities were measured in a microtiter infection assay as described (2). Briefly, two-fold, serial dilutions of each drug alone and in fixed-ratio combination with mismatched dsRNA were assayed in triplicate in 96-well microtiter plates. Cytolysis was measured via vital dye (neutral red) uptake by poly-L-lysine adherent cells as an endpoint for infection. Cells were incubated in the presence of drug dilutions for 1 hour prior to addition of virus. In the case of amphotericin B, both virus  
40 and cells were preincubated with drug prior to challenge. Cells were infected at a multiplicity of 0.1 so that endpoint cytolysis would be predominantly due to progeny virions synthesized in the presence of the drug. Percent protection was derived from  $A_{540}$  values of the dye in test wells relative to the difference in absorption between the cell control and virus control wells using the formula:

$$45 \quad \% \text{ Protection} = \frac{(\text{test minus virus})}{(\text{cells minus virus})}$$

50 **Calculation of Synergy** -Combined drug effects were calculated by the multiple drug analysis method of Chou and Talalay using the equation:

$$55 \quad CI = \frac{(D)_1}{(Dx)_1} + \frac{(D)_2}{(Dx)_2} + \frac{\alpha (D)_1 (D)_2}{(Dx)_1 (Dx)_2}$$

where CI is the combination index,  $(Dx)_1$  is the dose of drug 1 required to produce x percent effect alone, and  $(D)_1$  is the dose of drug 1 required to produce the same x percent effect in combination with  $(D)_2$ . The

values of  $(Dx)_2$  and  $(D)_2$  are similarly derived from drug 2. The value of  $\alpha$  is determined from the plot of the dose effect curve using the median effect equation:

$$fa/fu = (D/Dm)^m$$

where  $fa$  is the fraction affected by dose  $D$ ,  $fu$  is the unaffected fraction,  $Dm$  is the dose required for 50% effect and  $m$  is the slope of the dose-effect curve. For mutually exclusive drugs (i.e., similar mode of action), both drugs alone and their mixture give parallel lines in the median effect plot.

Mutually nonexclusive drugs (i.e., independent mode of action) will give parallel lines in the median effect plot but in mixture will give a concave upward curve. If the agents are mutually exclusive,  $\alpha$  is 0, and if they are mutually nonexclusive,  $\alpha$  is 1. Values obtained assuming mutual nonexclusiveness will always be slightly greater than mutually exclusive drugs. CI values of  $<1$  indicate synergy, values  $>1$  indicate antagonism and values equal to 1 indicate additive effects.

**Reverse Transcriptase Assay** -Reverse transcriptase activities in culture fluids were assayed in polyethylene glycol precipitates as described (3) using poly (A).(dT)<sub>15</sub> as template primer (Boehringer Mannheim) and 25  $\mu$ Ci [methyl-<sup>3</sup>H] dTTP (80.1 Ci/mmol, New England Nuclear) per reaction.

Review and analysis of these studies has given the following results and conclusions:

**Antiviral Activities** -The ability of each drug alone and in combination with mismatched dsRNA to protect target cells from HIV infection is shown in Table 1. With the exception of ribavirin, full protection was observed at all concentrations of each drug early in the incubation period immediately following cytolysis in the virus control (no effectors) wells. Virus-induced cytolysis at the lower doses of these drugs occurred one day later and assays were processed again at this time so that dose-dependent relationships could be achieved. Assays were also processed for ribavirin at this time, although full protection was never achieved at any sub-toxic concentration of this drug, even early in the infection process. The most effective ( $>10\%$  protection) concentrations of each drug produced greater anti-HIV activity in combination with mismatched dsRNA than when used alone. All drugs were non-toxic to MT-2 cells at the concentrations utilized in these studies.

Table 1.  
Effect of Various Antivirals in Combination with Mismatched dsRNA on HIV-Induced Cytopathicity.

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	10	20	40	80	160	320	640	1280	2560	5120	10240
0	0	9	19	19	20	23	28					
0.4	6	13										
0.8	17		24									
1.6	20			32								
3.2	16				44							
6.4	31					67						
12.8	28						74					

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	10	20	40	80	160	320	640	1280	2560	5120	10240
0	0	11	27	22	29	45	58					
0.4	12	31										
0.8	36		61									
1.6	44			60								
3.2	62				85							
6.4	71					100						
12.8	81						100					

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	7	14	28	56	112	224	448	896	1792	3584	7168
0	0	12	16	23	29	30	28					
0.4	9	14										
0.8	22		26									
1.6	38			51								
3.2	51				71							
6.4	22					84						
12.8	21						87					

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	0.02	0.04	0.08	0.16	0.32	0.64	1.28	2.56	5.12	10.24	20.48
0	0	5	3	5	8	17	30	58	100			
0.4	3	2										
0.8	1		1									
1.6	4			9								
3.2	7				23							
6.4	9					65						
12.8	10						84					
25.6	16							94				
51.2	35								100			

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2	102.4
0	0	6	9	14	21	35	52					
0.4	9	8										
0.8	18		12									
1.6	30			41								
3.2	33				92							
6.4	55					100						
12.8	65						90					

Mean percent protection at the following concentrations of effectors												
Mismatched dsRNA (µg/ml)	0	0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	25.6	51.2	102.4
0	0	3	6	10	14	26	58	90				
0.4	6	7										
0.8	16		21									
1.6	43			52								
3.2	57				81							
6.4	59					100						
12.8	78						97					
25.6	81							100				

**Multiple Drug Effects** -CI values for mismatched dsRNA in dual combination with eight other anti-HIV drugs at 50% and 95% protection values are given in Table 2. Various degrees of synergism were observed. The greatest degree of synergism was between mismatched dsRNA and rIFN- $\alpha$  where CI values were the lowest (0.01 to <0.01). The least amount of synergism was observed with amphotericin B where CI values were the highest observed and indicated more of an additive effect at 50% protection CI = 0.90 pr 1.08). Other drugs demonstrating synergism with mismatched dsRNA (CI values less than 1) were rIFN- $\beta$ , rIFN- $\gamma$ , azidothymidine, ribavirin, phosphonoformate, amphotericin B and castanospermine. Very little difference was observed if CI values were calculated on the assumption of mutual exclusiveness versus mutual non-exclusiveness for each drug tested.

Table 2.

CI Values for Combined Drug Effects with Mismatched dsRNA as a Core Drug

Drug	CI at Following % Protection Values*		
	50	90	95
rIFN- $\alpha$ A	.01 (.01)	<.01 (<.01)	<.01 (<.01)
rIFN- $\beta$	.34 (.36)	.14 (.14)	.10 (.10)
rIFN- $\gamma$	.55 (.56)	.37 (.37)	.33 (.33)
Azidothymidine	.55 (.56)	.40 (.40)	.37 (.37)
Ribavirin	.35 (.35)	.19 (.19)	.15 (.15)
Phosphonoformate	.58 (.63)	.65 (.68)	.70 (.72)
Amphotericin B	.90 (1.08)	.67 (.71)	.65 (.67)
Castanospermine	.55 (.61)	.21 (.23)	.16 (.17)

\*CI values were calculated from the data in Table 1. Values >1 indicate antagonism, <1 indicate synergism and equal to one indicate an additive effect. CI values calculated assuming mutual exclusiveness are given along with values obtained assuming mutual nonexclusiveness in parentheses.

**Virus Synthesis** -virus production in H9/HTLV-III<sub>g</sub> cultures was examined in the presence and absence of IFNs, mismatched dsRNA and the combination of these drugs (Table 3). Mismatched dsRNA alone (50  $\mu$ g/ml) had very little effect on the virus production (6% decrease) while rIFN- $\alpha$ A, rIFN- $\beta$ , and rIFN- $\gamma$  (500 IU/ml) inhibited virus production by 53%, 56%, and 20%, respectively. The presence of mismatched dsRNA resulted in a mild reduction in the inhibition of virus production induced by the IFNs, where this inhibition was decreased from 53% to 47% for rINF- $\alpha$ A, from 56% to 51% rINF- $\beta$ , and from 20% to 15% for rINF- $\gamma$ . Other studies (not shown) indicate these concentrations of mismatched dsRNA and rIFNs alone or in

combination had no effect on cell division.

TABLE 3.

## Effect of Mismatched dsRNA on IFN-Induced Inhibition of HIV Production

Effector*	cpm RT/ml (x 10 <sup>-3</sup> )	Avg. cpm RT/ml (x 10 <sup>-3</sup> )	% Decrease
Control - 1	877	910	-
Control - 2	943		
Mismatched dsRNA - 1	896	852	6
Mismatched dsRNA - 2	807		
rIFN- $\alpha$ A - 1	426	428	53
rIFN- $\alpha$ A - 2	430		
rIFN- $\beta$ - 1	396	398	56
rIFN- $\beta$ - 2	400		
Mismatched dsRNA + rIFN- $\alpha$ A - 1	418	479	47
Mismatched dsRNA + rIFN- $\alpha$ A - 2	540		
Mismatched dsRNA + rIFN- $\beta$ - 1	411	448	51
Mismatched dsRNA + rIFN- $\beta$ - 2	485		
Control - 3	1,945	2,013	-
Control - 4	2,081		
rIFN- $\gamma$ - 1	1,508	1,602	20
rIFN- $\gamma$ - 2	1,696		
Mismatched dsRNA + rIFN- $\gamma$ - 1	1,669	1,704	15
Mismatched dsRNA + rIFN- $\gamma$ - 2	1,738		

\* Duplicate cultures with equal densities of washed H9/HTLV-III<sub>B</sub> cells were incubated in the presence and absence of effectors for 48 hours. Conditioned culture fluids were then harvested and assayed for reverse transcriptase activity. Recombinant IFN- $\gamma$   $\pm$  mismatched dsRNA was tested in a second experiment and therefore had a separate set of controls. Mismatched dsRNA = 50  $\mu$ g/ml, IFNs = 500 IU/ml.

The synergies observed in this study between mismatched dsRNA and five classes of anti-HIV drugs suggest that mismatched dsRNA may have a powerful and versatile role as a core drug in combination therapy for ARC and AIDS. Double-stranded RNAs, including mismatched dsRNA, activate IFN-induced enzymes involved in the establishment of an antiviral state, including 2,5-oligoadenylate synthetase and a ribosome-associated protein kinase. I have observed that 2,5-oligoadenylates inhibit retroviral reverse transcriptases, a finding which suggests that activation of 2,5-oligoadenylate synthetase by mismatched dsRNA may represent a unique mechanism for antiviral activity against viruses that require reverse transcription for replication. Most importantly, not all of the pleiotropic activities of IFNs appear to be shared with mismatched dsRNA. This is exemplified by the fact that flu-like side effects of IFN therapy are not present during mismatched dsRNA therapy, as well as the finding that mismatched dsRNA neither inhibited HIV production as did IFN, nor potentiated this activity of IFN's (see Table 3). These latter results are in contrast to the synergism observed between mismatched dsRNA and the IFNs on establishing an antiviral state (see Table 2), further suggesting that these drugs have common as well as distinct pathways for antiviral activity.



Another class of anti-HIV drugs, the inhibitors of reverse transcription, I have also found to be synergistic with mismatched dsRNA. A broad family of 2',3'-dideoxynucleoside analogues can be metabolized to become potent inhibitors of retroviral reverse transcriptase. For example, R. Yarchoan and S. Broder (New England Journal of Medicine, February 26, 1987, volume 316, pages 557-564) describe two dideoxynucleosides designated 3'-azido-2'-deoxythymidine (AZT) and 2',3'-dideoxycytidine (DDC) which are analogues of the naturally utilized thymidine and 2'-deoxycytidine, respectively. See also U.S. Patent No. 4,724,232 describing the use of AZT in the treatment of ARC and AIDS in amounts of from 5 to 250 mg per kilogram body weight of recipient per day. There is evidence that phosphorylated nucleosides inhibit retroviruses by acting as chain terminators such that the viral reverse transcriptase is fooled into adding these analogues to the growing chain of DNA; this blocks further formation of the 5'-3' phosphodiester bond thereby resulting in premature DNA chain termination. Similar effects may be seen with purine analogues such as 2',3'-dideoxyadenosine. Unfortunately, these various pyrimidine and purine analogues can also inhibit various normal cell enzymes, such as DNA polymerase alpha found in bone marrow and other organs. These inhibitions of normal cell functions thus lead to various profound toxicities. For example, chronic administration of AZT causes severe anemia and leukopenia in more than 50% of cases; accordingly, many AZT treated patients require regular blood transfusions and may actually succumb to the side-effects of bone marrow damage, already weakened by their prior retrovirus infection.

The two inhibitors used in this study were azidothymidine and foscarnet. Azidothymidine, a thymidine analog, becomes phosphorylated intracellularly and is incorporated into nascent DNA where it causes premature chain termination. Phosphorylated azidothymidine is utilized by reverse transcriptase 100 times more effectively than by cellular DNA polymerases, thus allowing a seemingly large window of selectivity. Phosphonoformate, (Foscarnet) another inhibitor of reverse transcription, has strong anti-HIV activity *in vitro* in addition to selectively inhibiting influenza virus RNA polymerase and herpes virus DNA polymerase. Both of these drugs demonstrated potent, selective inhibition of HIV in the microtiter infection assay. Their observed synergism with mismatched dsRNA suggests that such synergism may also be observed with other inhibitors of reverse transcription.

dsRNAs in general and Ampligen in particular can substantially reduce by at least 5-fold the concentration of AZT required for significant virustatic activity *in vitro*. Moreover, at the higher concentrations of AZT tested, there is a synergistic relationship between the two compounds. Therefore, Ampligen will allow a decreased effective therapeutic dose of AZT *in vivo* with a concomitant decrease in AZT-associated toxicity.

Since the two drugs act by entirely different modes of action, they will demonstrate *in vivo* no toxicities other than those associated with each drug alone. Indeed, I observed no evidence of synergistic toxicity clinically even when Ampligen was combined with more closely allied molecules such as the interferons. Moreover, since Ampligen has demonstrated clinically in my studies various immunomodulatory activities in addition to its antiviral properties (both possibly mediated through similar mechanisms such as the 2'-5' oligo A pathway), the use of Ampligen in conjunction with AZT may have pronounced and long-term beneficial effects on the course of HIV infections well beyond that which I now report *in vitro*.

The importance and specificity of my discovery is no better illustrated than in light of the report on the combination of AZT with another powerful anti-AIDS drug, termed ribavirin, which appeared in Science, March 13, 1987, from Dr. Hirsch's laboratory at Harvard: they report profound antagonism when ribavirin is added to AZT in order to achieve therapeutic potentiation. That is, other drugs, when added to reverse transcriptase inhibitors, actually erode their therapeutic potential rather than enhancing it.

The synergistic combination of dsRNAs and inhibitors of reverse transcriptase are also useful in the treatment of retrovirus-induced cancer tumors.

Ribavirin may represent a class of anti-HIV drugs which interfere with 5'-mRNA processing. Although the mechanism of antiviral activity of ribavirin is not clear, this drug is thought to compete with quanosine in the formation of mRNA cap structures and/or interfere with the functional methylation of these molecules. Other inhibitors of functional HIV mRNAs, such as antisense DNAs, should also exhibit synergy of this anti-HIV mechanism with mismatched dsRNA.

Amphotericin B, a polyene macrolide antifungal antibiotic which interacts with sterols and binds to them irreversibly, represents yet another unique class of agents that are active against a variety of lipid-enveloped viruses, including HIV. Although amphotericin B exhibits severe *in vivo* toxicities, the methyl ester form of this drug also exhibits anti-HIV activity and has a low cellular toxicity profile *in vitro*. Therefore, amphotericin B methyl ester will be more beneficial in combination therapy with mismatched dsRNA than the parent compound.

The final agent demonstrating synergism with mismatched dsRNA was castanospermine. Castanosper-

mine is a plant alkaloid which inhibits glycoprotein processing, and was investigated because of the envelope of HIV contains two heavily glycosylated proteins. gp120 as an outer membrane glycoprotein and gp41 as a transmembrane glycoprotein. Interaction between gp120 and the OKT4 surface antigen of T cells, which acts as receptor for the virus, is partly responsible for the cellular tropism of HIV. Recent studies by others with glycolases and lectins have shown that protein glycosylation plays an important role in gp120-LKT4 interaction and HIV infection. Maturation of glycoproteins is dependent on a series of enzymes for the processing of carbohydrate moieties which usually results in the transformation of high-mannose to complex-type oligosaccharides. Castanospermine inhibits glucosidase I which results in a high-mannose type N-glycosylated protein. Under conditions of infection by progeny virions synthesized in the presence of castanospermine, the infectivity of HIV was attenuated (see Table 1). This attenuation was independent of any effect that castanospermine may have had on the state of receptor glycosylation and was actually due to a reduction in infectious virus yield, as determined by TCID-50 assays, with no effect on overall virus production as measured by virus-associated reverse transcriptase activity. The finding that this anti-HIV activity is synergistic with mismatched dsRNA (Table 2) suggests that mismatched dsRNA will be synergistic with agents which interfere with HIV receptor binding. These agents will include neutralizing antibodies, blocking peptides such as peptide "T", or other inhibitors of glycoprotein processing.

#### Table of References

1. Elbein, A.D. (1987) Inhibitors of the Biosynthesis and Processing of N-linked Oligosaccharide Chains. *Ann. Rev. Biochem.* 56,497-534
2. Montefiori, D.C., Robinson, W.E., Jr., Schuffman, S.S. and Mitchell, W.M. (1987) Evaluation of Antiviral Drugs and Neutralizing Antibodies Against Human Immunodeficiency Virus by a Rapid and Sensitive Microtiter Infection Assay. *J. Clin. Microbiol.* (In press)
3. Poiesz, B.J., Ruscetti, F.W., Gazder, A.F., Bunn, P.A., Minna, J.D. and Gallo, R.C. (1980) Detection and Isolation of Type C Retrovirus Particles From Fresh and Cultured Lymphocytes of a Patient With Cutaneous T-cell Lymphoma. *Proc. Natl. Acad. Sci. USA* 77, 7415-7419.

#### Claims

1. Product containing dsRNA and an antiviral agent as a combined preparation for simultaneous, separate or sequential use in the treatment of a viral disease by inhibition of viral activity, viral expression or both.
2. The product of claim 1, which is a therapeutic composition comprising dsRNA and the antiviral agent.
3. The product of claim 1 or claim 2, wherein the antiviral agent is a cytokine, an inhibitor of reverse transcriptase, a lipophile or a glycoprotein processing inhibitor.
4. The product of claim 3, in which the antiviral agent is rIFN- $\alpha$ , r-IFN- $\beta$ , rIFN- $\gamma$ , azidothymidine, phosphorofornate, ribavirin, amphotericin B or castanospermine.
5. The product of claim 4, wherein the antiviral agent is 3'-azido, 3'-deoxythymidine or a pharmaceutically acceptable salt thereof.
6. The product of any one of claims 1 to 5, wherein the dsRNA is mismatched dsRNA.
7. The product of claim 6, wherein the dsRNA is of the general formula  $r(n(C_{11-14}U))_n$ .
8. The product of claim 6, wherein the dsRNA is of the general formula  $r(n(C_{29}, G))_n$ .
9. The product of any one of claims 6 to 8, wherein the dsRNA contains regions of bond breakage.
10. The product of claim 1 or claim 2, wherein the dsRNA is Ampligen and the antiviral agent is 3'-azido, 3'-deoxythymidine.

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☒ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**